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Award Number: DAMD17-02-1-0401

TITLE: Isolation and Analysis of Human Kekkon-Like Molecules, a Family of Potential Inhibitors of ErbB Receptor Tyrosine Kinases

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REPORT DATE: April 2004

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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20040901 087

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 2004	Annual Summary (1 Apr 2003 - 31 Mar 2004)

4. TITLE AND SUBTITLE
Isolation and Analysis of Human Kekkon-Like Molecules, a Family of Potential Inhibitors of ErbB Receptor Tyrosine Kinases

5. FUNDING NUMBERS
DAMD17-02-1-0401

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Purpose: Identification and characterization of proteins involved in intrinsic negative feedback loops autoregulating the transforming c-ErbB2/EGF Receptor activity in vivo.
Scope: Receptor Tyronsine Kinase (RTK) activation trigger two distinct signal transduction cascades: Mitogenic Ras dependent MAP Kinase activation and PI3 Kinase dependent Akt/PKB survival signaling. To date, the regulatory circuits in the Ras signaling pathway are well described. In contrast, no genes involved in the feedback regulating of the PI3K - Akt/PKB signaling branch are known so far. To this end, a cell based genome wide screen employing double stranded RNA interference (dsRNAi) as well as an analysis of Akt mediated transcriptional response using DNA microarrays covering the whole genome have been initiated.
Progress: A quantitative assay to measure amounts of phosphorylated ("active") Akt has been established. The description and analysis of regulatory feedback regulation within the Akt signal transduction pathway using dsRNAi, chemical inhibitors and metabolic perturbation ahs been accomplished. The first genome wide dsRNAi screen for activators of Akt has been completed in replicates. Secondary screens to retest the results form the high-throughput efforts are developed. The data analysis is ongoing.

14. SUBJECT TERMS
Cell signaling, Receptor Tyrosine Kinase Inhibtor, Transcriptional Feedback Inhibition, Molecular Diagnostic Marker, Therapeutic Agent

15. NUMBER OF PAGES
12

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction

Background

Tumorigenesis is a multistep process culminating in the transformation of normal cells into highly proliferating, apoptosis resistant and malignant derivatives. Activating mutations or gene amplification of Receptor Tyrosine Kinases (RTK) facilitate a self-sufficiency in growth signals and provide a strong survival signal (1).

A high percentage of breast tumors are characterized by overexpression of the HER2/Neu receptor, a RTK (2,3,4) that potently activates PI3K and Ras dependent signal transduction pathways (5,6,7,8,9).

Studies performed in *Drosophila* have revealed a tight temporal and spatial control of EGFR pathway activity by negative feedback circuits (10,11). Based on the significant molecular conservation of the signal transduction machinery between man and the fly, it was hypothesized that negative feedback circuits regulating c-ErbB2 activity are present in humans. Additionally, this intrinsic feedback control might be inactivated by mutation in breast cancer patients, thereby contributing to the poor clinical outcome of c-ErbB2 positive breast cancers.

Goal

The purpose of this work is the identification and characterization of proteins involved in intrinsic negative feedback loops autoregulating Receptor Tyrosine Kinase activity in *vivo*. Currently, no genes involved in feedback regulation of the PI3 Kinase - Akt/PKB signaling branch have been described yet, although the presence of feedback regulation has been evident in *Drosophila* (12,13). In order to identify regulators of Akt activity, a cell based genome wide screen employing double stranded RNA interference (dsRNAi) has been initiated.

Significance

Diagnosis of c-ErbB positive breast cancer correlates with a poor overall survival rate of patients. This identifies the family of ErbB RTKs as an important therapeutic target. Proteins that down-regulate the transforming activity of ErbB Receptor Tyrosine Kinases should either represent interesting drug targets or potentially act as efficient therapeutics on their own.

Body

A *Drosophila* phospho-specific Akt Antibody

In collaboration with Cell Signaling, Beverly, MA, an antibody against Serine 505 of *Drosophila* Akt (homologous to Ser 473 of human Akt1) has been generated (P-dAkt^{Ser505}). The Antibody is highly specific: Stimulation of cells with Insulin induces a strong signal on Western Blots and in immunohistochemical staining, which can be suppressed by either the PI3 Kinase inhibitors Wortmannin and LY294002, or dsRNAi against Insulin Receptor (InR), Insulin Receptor Substrate 1-4 ortholog (Chico), PI3 Kinase and Akt itself.

Phosphorylation of Ser 505 closely follows the activity of Akt (14); making this Antibody the tool of choice to analyze Akt activity and its modulation by dsRNA mediated interference (dsRNAi) against single genes or gene pools (15).

A Pilot dsRNAi screen for regulators of Akt

In order to use the Antibody as a screening tool for libraries of double stranded RNA interference (dsRNAi) treated cells, a high throughput protocol using 384-well microtiter plates for culturing, dsRNA treating, P-dAkt^{Ser505} antibody staining and microscopic analysis has been established. Treatment of cultured *Drosophila* cells with gene-specific dsRNA has been shown to exclusively deplete the mRNA of the targeted gene, concomitant with a sharp decrease of the related protein level (15).

Initially, 94 genes encoding for Kinases, Phosphatases, small GTPases as well as other signaling molecules have been selected and tested as a pilot screen (please see my 2003 report of details). Image acquisition was facilitated by an Autoscope, a microscope with a motorized stage and automated image acquisition software. Image analysis was performed "by eye".

While successful to a certain extend, the pilot screen showed the absolute necessity for a faster and more quantitative assay if full genomes with 21.500 dsRNAs were to be screened in replicates, especially when the screens are done with various different stimuli (e.g. absence or presence of Insulin stimulus, genome wide dsRNA modifier screens).

A fast and quantitative cell based high throughput assay: The Cytoblot / In Cell Western

The Cytoblot / In Cell Western is a chimera combining parts from immunohistochemical staining of tissue culture cells and simple western blot techniques into a high throughput format (16).

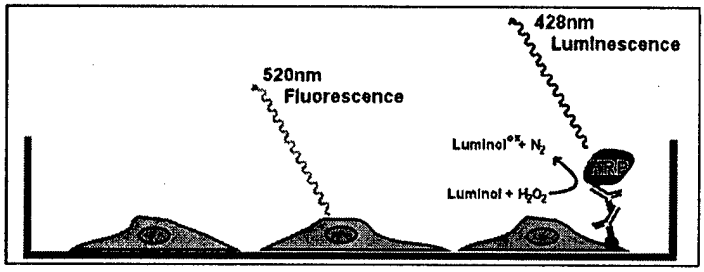

Figure 1: The Cytoblot

Cells are seeded in a 384 well plate and grown for 3 days. Cells are fixed and stained with a combination of anti P-Akt, HRP coupled secondary Antibody and a DNA dye (show in green). Data is acquired in a 384 well plate reader

The cells are seeded in 384 well plates and grown in the presence of various dsRNAs to confluency for a three day period. Then, the cells are fixed and immunohistochemically stained with anti –P Akt antiserum. However, instead of a fluorophor-conjugated secondary antibody, Horseradish Peroxidase conjugated secondary Antiserum is used, very much alike standard Western Blotting. Detection of P-Akt is facilitated in a 384 well

plate reader quantifying the chemiluminescence emitted after addition of Western Blot substrate.

The individual dsRNAs can influence the luminescent signal in two ways: Either the dsRNA targets and represses the expression of a gene required for the phosphorylation or dephosphorylation of Akt. Alternatively, the dsRNA is eliciting an effect on the cell cycle / cell death / cell adhesion machinery, leading to an altered cell number and consequently an altered chemiluminescent signal from this well (17). The former dsRNAs are the "specific hits" the screen is designed for, the latter would represent a distinct form of "noise". In order to avoid false positive hits caused by cell number variation, the luminescence from each well is normalized to a cell count value of the very same well. This is facilitated by fluorescently staining nuclear DNA and the subsequent recording of the fluorescent value in a 384 well plate reader. Cell number titration experiments showed that the fluorescent values are proportional to the number of cells in each well.

The Cytoblot is a fast and robust assay. While automated microscopy of a single plate takes 2.5 – 3 hrs, a dual Luminescence and Fluorescence read takes 6 minutes. Furthermore, microscopic analysis acquires data from a fraction of a single 384 well surface, while the plate reader based Cytoblot reads >90% of the well.

Statistical analysis of the Cytoblot / In Cell Western data

My initial idea of normalization aimed at a simple linear relationship of cell number per well (represented by the value obtained from fluorescent reads of the nuclear stain) and the value of measured chemiluminescence, indicating the amount of phosphorylated Akt per well. In such a model, the fraction of chemiluminescence and nuclear fluorescence should be constant when non-RNA treated wells of various cell densities are analyzed, regardless of cell number.

Unfortunately, this is not the case. The results of such an operation are very high values for sparsely populated wells and very low values for confluent wells. This resulted in a large number of false positive hits in test screens: All dsRNAs which inhibited cell growth scored very high when put into relation to the median of the 384 well plate. (Score: Divergence of a single well chemiluminescent / nuclear fluorescence ratio from the Median of the 384 well plate).

Currently, I am normalizing by the k-nearest neighbor method (18): Chemiluminescence values of individual wells (representing amounts of phosphorylated Akt) are compared to the Median of chemiluminescence of 10 wells with similar or equal nuclear fluorescence (cell density). The output is an intensity (= cell number) weighted Score, which effectively compensates the non-linear relationship of chemiluminescence and nuclear fluorescent intensities.

In order to facilitate a more sophisticated statistic analysis and modeling of the process, I recently entered a collaboration with Xiaochun Li and Robert Gentleman in the Biostatistics Department at the Harvard School of Public Health.

Evaluation of the Cytoblot / In Cell Western assay

In order to test the reliability of the Cytoblot / In Cell Western assay and its analysis, I tested my assay system with dsRNAs against known members of the Akt signaling pathway: The RTK Insulin Receptor, IRS 1-4 ortholog Chico, PI3 Kinase 92E, Akt itself, and Tsc1 (Hamartin ortholog), Tsc2 (Tuberin ortholog), the GTPase Rheb and S6K. All dsRNAs were present in seven replicates, the experiment was performed under serum starved, non-stimulated condition as well as with a 10 minutes Insulin stimulus.

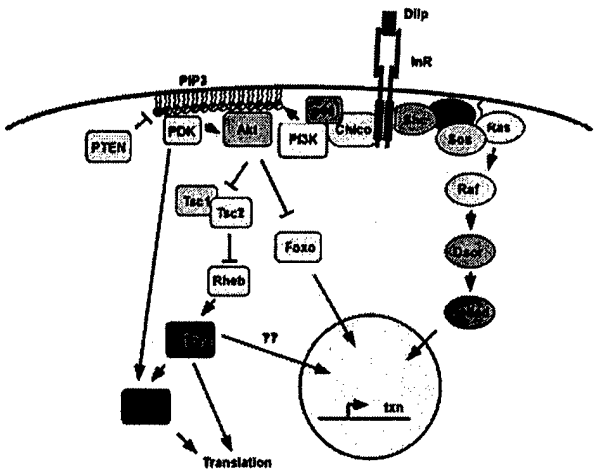

Figure 2: The Akt Signal Transduction Pathway

Activation of the RTK (shown here: Insulin Receptor) results in IRS (Chico) - PI3K – PIP3 dependent activation of Akt. Pten negatively regulates Akt activity. Downstream of Akt, Tsc2 is inhibited by Akt mediated phosphorylation, which results in de-repression of the small GTPase Rheb, resulting in dTor mediated S6K activation.

Employing a cutoff at a score of +/- 3, all known components of the pathway were recognized by the assay / analysis. Positive effector molecules located upstream of Akt (InR, PI3K, Chico) blocked Insulin dependent Akt phosphorylation ($P < 1E-05$) while they did not score in the not-stimulated case. dsRNA against the negative effector Pten showed the reverse phenotype: It scored in the absence of Insulin stimulus as suppressor ($P < 2E-08$) and did not show any additional induction of P-Akt when Insulin was present.

In summary, the assay / analysis is well capable of detecting known regulators of Akt phosphorylation. Activators and inhibitors upstream of Akt give complementary phenotypes, consistent with the current literature (19). Therefore, this method constitutes a valuable assay to evaluate P-Akt levels in the context of dsRNAi mediated gene "knock down".

Analysis of negative feedback signaling in the Akt signal transduction pathway

Surprisingly, Akt phosphorylation is sensitive to the interference of downstream components of the pathway. On the first glance this is counterintuitive. Phosphorylation of Akt represents in input into Akt (regulated by upstream elements of the signal transduction pathway) and not an output mediated by downstream regulators.

However, dsRNAi against downstream mediators required positively for Insulin signal transduction like the small GTPase Rheb and S6K induce ectopic phosphorylation (activation) of Akt, regardless if Insulin is present or not. Conversely, RNAi against the negative regulators Tsc1 and Tsc2 always suppress the P-Akt signal, even (although weakly) in the non stimulated state.

The simplest interpretation is the generation of an inhibitory feedback signal mediated by the components downstream of Akt, namely Rheb, Tsc1/2 and S6K. In case the negative feedback is disrupted, ectopic activation of Akt occurs.

In order to test this hypothesis by different means than dsRNAi, dTor, an activator of S6K, was inhibited by two different measures: Chemically, by exposing the cultured cells to the small molecule Rapamycin, which is an effective inhibitor of dTor (20,21,22); and metabolically, by starving cultured cells in amino acid free media, thereby potently inhibiting dTor activity (12,13). The outcome of these experiments confirm the previous dsRNA interference results. Metabolic starvations as well as Rapamycin induced dTor inhibition lead to a significant increase in P-Akt, validating the idea of a negative feedback loop auto-regulating the activity of the pathway after stimulation.

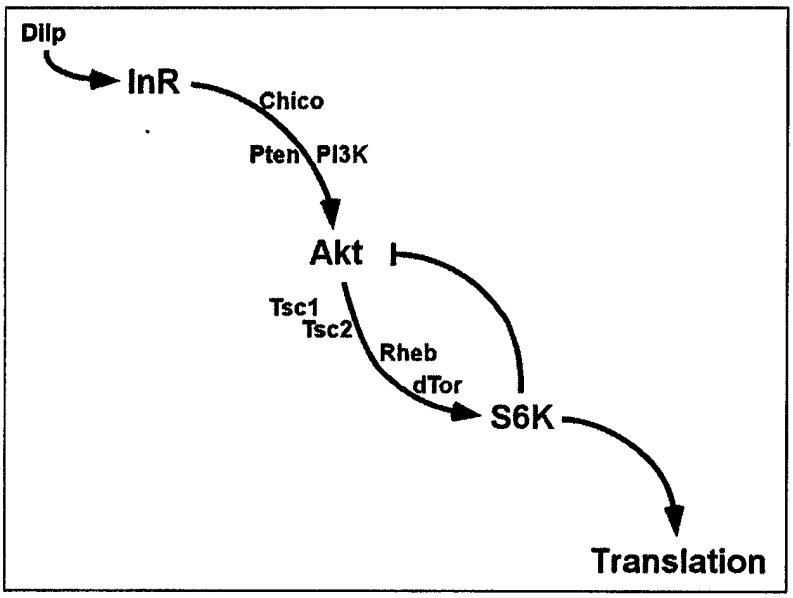

Figure 3: Feedback regulation of Akt.

Akt becomes activated by an RTK dependent stimulus (here: Insulin Receptor). In turn, this leads to the generation of an inactivating stimulus signaling back to Akt. Inactivation of positive mediators of downstream Akt signaling like Rheb, dTor and S6K results in the disruption of the feedback and ectopic activation of Akt. Conversely, activation of downstream signaling by disrupting Tsc1 and Tsc2 lead to permanent feedback inhibition and suppressed Akt phosphorylation after an Insulin stimulus. Positive regulators are marked in red, inhibitory components are depicted in blue.

The open and very interesting questions remaining open are what other components are involved in mediating the feedback inhibition and what mechanism(s) is/are involved? In order to tackle these questions, I will pursue genome wide dsRNAi screens and additional experiments using chemical inhibitors to decipher the molecular mechanism which relays the feedback to Akt.

A genome wide dsRNA collection

A unique collection of 21.500 dsRNAs, covering all genes annotated in the *Drosophila* genome, has been established in the laboratory of Prof. Norbert Perrimon (17). Treatment of cultured *Drosophila* cells with gene-specific dsRNA has been shown to exclusively deplete the mRNA of the targeted gene, concomitant with a sharp decrease of the related protein level (15). The combination of treating *Drosophila* KC_{167} cells with dsRNA against every single gene with immunohistochemical staining against phosphorylated/activated Akt allows the systematic identification of regulators of Akt.

A genome wide dsRNAi screen for suppressors of Akt phosphorylation

At this stage, I have carried out two replicates of a genome wide dsRNAi using the P-Akt specific Antibody on non-stimulated cells. As these experiments were just finished this week, an analysis has not been performed yet. This screen will be followed by the screening two replicates of Insulin stimulated genome wide dsRNA sets.

Analysis of the transcriptional response to Insulin

In a group effort of the laboratories of Norbert Perrimon, Alan Michelson and the Harvard Partners Center for Genetics and Genomics, we previously in-house produced DNA microarrays containing spotted PCR products of 13 600 open reading frames of the Drosophila genome. I established fluorescent mRNA labeling and microarray hybridization protocols and developed a data treatment scheme to write the normalized results into contingency tables in order to facilitate analysis with various clustering tools.

The goal of the analysis was to characterize a group of target genes which respond to an Insulin signal. In this group of genes, molecules involved in the regulatory feedback inhibition might be found.

An initial analysis by western blot using a phospho-specific antibody against Akt, an essential downstream mediator of the Insulin Receptor showed that the *Drosophila* cell lines used (SL2 and KC_{167}, embryonic hemocyte derived cells) are in fact insulin responsive. Serum starved cells showed only a basal level of Akt phosphorylation, while Insulin elicited a robust increase in phosphorylated Akt.

In a first series of hybridizations, I compared mRNA pools from SL2 cells which were serum starved for 20 hours to mRNA pools from cells which were first serum starved for 20 hours, followed by stimulation with Insulin for 30 minutes, 1 hour, 2 hours and four hours. The same experiment was replicated using a different *Drosophila* cell line, KC_{167}. The time course was chosen short deliberately, in order to avoid indirect influences by immediate early gene products.

However, analysis of the data revealed only minor, insignificant changes in the transcriptional profiles of stimulated vs. unstimulated cells within the time frame observed. In order to exclude those endogenous basal levels of Insulin signaling results in an already activated transcriptional response which can not be further modified, I additionally treated the serum staved, not stimulated cells with 800nM Wortmannin, a PI3 Kinase inhibitor, for 4 hours. Comparison of the Wortmannin treated, serum starved mRNA pool to the Insulin treated mRNA pool did not uncover any significant changes of transcriptional profiles.

After this discouraging experience, I initially considered the discontinuation of this line of experiments. However, in the light of the analysis of the feedback regulation within the Akt pathway, experiments are on its way to test the transcriptional response to Akt activation when the negative feedback loop is interrupted either chemically (Rapamycin treatment) or by the means of dsRNAi.

Key Research Accomplishments

- A phospho-specific antibody against phosphorylated Ser505 of *Drosophila* Akt has been characterized and tested on western blotting and immunohistochemistry employing dsRNAi and chemical inhibitors.
- Quantitative high throughput protocol for screening dsRNAi treated tissue culture cells by Cytoblot / In Cell Western established. Method to normalize against cell number variation implemented.
- Statistical analysis for scoring and ranking the effect of individual dsRNAs in place
- Description and analysis of regulatory feedback regulation within the Akt signal transduction pathway using dsRNAi, chemical inhibitors and metabolic perturbation.
- Completion of the first two replicates of genome wide dsRNAi screens for regulators and components of Akt phosphorylation (non-stimulated case). The genome wide dsRNAi screens under Insulin-stimulated condition are scheduled for late April.
- Co-production of a *Drosophila* genome wide, PCR based DNA microarray and establishment of all required protocols and data analysis tools
- Experiments profiling the genome wide transcriptional response to Akt/PKB activation in order to isolate genes involved in feedback regulation are under way

Reportable outcomes

None yet.

Preliminary conclusions and outlook

Alterations of expression and/or activity of several components of the Akt signal transduction pathway are molecularly linked to proliferative diseases and especially breast cancer (21,22). The Tor inhibitor Rapamycin and its derivatives are currently investigated in clinical trials as potential cancer drugs (22). It is therefore important to notice that disruption of Tor function by Rapamycin might not only disrupt the downstream events of signal transduction, but counterproductively might also ectopically activate Akt by suppressing feedback inhibition on Akt, with all its consequences to elevated cell survival (1).

Continuing this line of work described here will provide additional molecular targets required to compensate against this effect and will lead to additional valuable knowledge to fight breast cancer.

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